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Invasion-Inhibitory Antibodies Elicited by Immunization with *Plasmodium vivax* Apical Membrane Antigen-1 Expressed in *Pichia pastoris* Yeast

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In a recent vaccine trial performed with African children, immunization with a recombinant protein based on *Plasmodium falciparum* apical membrane antigen 1 (AMA-1) conferred a significant degree of strain-specific resistance against malaria. To contribute to the efforts of generating a vaccine against *Plasmodium vivax* malaria, we expressed the ectodomain of *P. vivax* AMA-1 (PvAMA-1) as a secreted soluble protein in the methylotrophic yeast *Pichia pastoris*. Recognized by a high percentage of sera from individuals infected by *P. vivax*, this recombinant protein was found to have maintained its antigenicity. The immunogenicity of this protein was evaluated in mice using immunization protocols that included homologous and heterologous prime-boost strategies with plasmid DNA and recombinant protein. We used the following formulations containing different adjuvants: aluminum salts (Alum), *Bordetella pertussis* monophosphoryl lipid A (MPLA), flagellin FliC from *Salmonella enterica* serovar Typhimurium, saponin Quil A, or incomplete Freund's adjuvant (IFA). The formulations containing the adjuvants Quil A or IFA elicited the highest IgG antibody titers. Significant antibody titers were also obtained using a formulation developed for human use containing MPLA or Alum plus MPLA. Recombinant PvAMA-1 produced under "conditions of good laboratory practice" provided a good yield, high purity, low endotoxin levels, and no microbial contaminants and reproduced the experimental immunizations. Most relevant for vaccine development was the fact that immunization with PvAMA-1 elicited invasion-inhibitory antibodies against different Asian isolates of *P. vivax*. Our results show that AMA-1 expressed in *P. pastoris* is a promising antigen for use in future preclinical and clinical studies.

The pursuit of a *Plasmodium vivax* vaccine remains a great challenge. Furthermore, despite the widespread distribution of the disease worldwide and increasing reports of morbidity and mortality, research on *P. vivax* malaria has been neglected for many years (1, 2). In spite of its importance and in contrast to *Plasmodium falciparum* malaria, only three clinical trials based on subunit *P. vivax* vaccines have been completed to date (http://www.clinicaltrials.gov/).

One of the leading candidates for the development of a vaccine against malaria is the transmembrane protein apical membrane antigen-1 (AMA-1), which is characteristic of *Plasmodium* sp. and formed by a cysteine-rich ectodomain, transmembrane region, and C-terminal region (3). AMA-1 is initially expressed in sporozoites (4); at the end of asexual reproduction inside hepatocytes or erythrocytes, the expression of AMA-1 increases and the protein is translocated to the micronemes in the apical pole (5). Recent studies have shown that the hydrophobic regions located in domain II of *P. falciparum* AMA-1 bind to rhoptry neck protein 2 (RON2) (6) to form a complex, a process that is inhibited by antibodies (7) and peptides (8), thereby preventing invasion. These data suggest that the AMA-1–RON complex is essential for parasite invasion. Although experiments with conditional gene deletion have confirmed that AMA-1 is required for merozoite invasion of red blood cells, it has been found to be dispensable for sporozoite invasion of hepatocytes (9).

Many significant variations (alleles) have been observed in *P. falciparum* and *P. vivax* isolates (10–17). The majority of *P. vivax* AMA-1 (PvAMA-1) polymorphisms are described in domain I (13–15), whereas domain II is more conserved, suggesting an important function (16, 17).

A number of phase II clinical trials using recombinant proteins or viruses based on *P. falciparum* AMA-1 (PfAMA-1) have been performed to date (18–21). Recently, a vaccine trial was conducted with 400 African children using the malaria vaccine FMP2.1/AS02A. This vaccine is a recombinant prokaryotic protein based on PfAMA-1 from the 3D7 strain of *P. falciparum* and is administered as a formulation containing the adjuvant system AS02A (oil-in-water emulsion with 3-deacylated-monophosphoryl lipid A from *Salmonella enterica* serovar Minnesota and a highly purified saponin, QS-21). However, the results of the pri-

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mary analyses revealed an efficacy against malaria of only 17.4%. Due to the possibility of strain-specific immunity, a secondary analysis was performed and described a much higher efficacy (64.3%) against malaria caused by parasites with the *pfama-1* gene, corresponding to the 3D7 strain. This result led to the conclusion that vaccination with FMP2.1/AS02A elicited a significant strain-specific resistance against *P. falciparum* malaria (20).

Very recently (in 2013), the results of clinical trials were published on genetic immunization with the *pfcsp* and *pfama-1* genes in a heterologous prime-boost vaccination regimen. This protocol consisted of priming with recombinant plasmid DNA, followed by a booster immunization with human type 5 replication-deficient adenovirus (AdHu5), both expressing the *pfcsp* and *pfama-1* genes from *P. falciparum* strain 3D7. The results showed that 27% of the individuals were sterilely protected upon experimental challenge by exposure to the bite of mosquitos infected with the homologous parasite strain (22).

In previous studies, we have shown that recombinant proteins based on *P. vivax* AMA-1 are immunogenic in natural infection (23–26). Furthermore, a prime-boost strategy using recombinant AMA-1 administered in Montanide ISA720, followed by booster injection of AdHu5 expressing PvAMA-1, produced high titers of long-lasting antibodies and specific memory T cells (27).

The disadvantage of prokaryotic systems for recombinant protein production is the fact that the protein based on PvAMA-1 representing the entire ectodomain was insoluble (26). In spite of efforts toward the standardization of an efficient protocol for solubilization/refolding, the yield was low, and endotoxin contamination was reported (23, 26). In addition, the recognition of conformational epitopes may be critical for protective antibodies. Accordingly, the expression of recombinant proteins using eukaryotic systems may represent a long-term advantage in an effort to solve these problems. Indeed, a previous study expressed the PvAMA-1 ectodomain in *Pichia pastoris*, and this antigen was immunogenic in rhesus monkeys when administered with the SBSA2 adjuvant (10).

Based on the promising results of vaccination with PfAMA-1 described above, we expressed and characterized the immunogenic properties of recombinant PvAMA-1 expressed as a soluble protein in the yeast *P. pastoris*, aiming at the development of a vaccine against *P. vivax* malaria.

MATERIALS AND METHODS

Synthesis, cloning, and yeast expression. The synthetic gene encoding amino acids 43 to 487 of the PvAMA-1 ectodomain was synthetized by GenScript USA, Inc. (Piscataway, NJ) with codon optimization to improve expression in *P. pastoris*. The amino acid sequence was based on that of a Brazilian *P. vivax ama-1* isolate (23). Three potential N-glycosylation sites were altered to prevent unwanted glycosylation (178N→S, 226N→D, and 441N→Q) by using substituent amino acids from other available AMA-1 sequences of malaria parasites (10). The constructs were designed with appropriate restriction sites and a carboxyl-terminal His_6 tag to enable purification. The synthetic gene cloned in the pUC57 vector was removed by digestion with an NotI enzyme mix (New England BioLabs) and subcloned into the NotI site of the *P. pastoris* expression vector pPIC9K (Invitrogen). This expression vector contains the nucleotide sequence encoding the α-factor signal peptide of *Saccharomyces cerevisiae* for protein secretion, the AOX1 promoter for the control of gene expression, and the HIS4 gene for selection of the recombinant yeast clones. A clone was selected containing the *pvama-1* gene in the correct orientation. The plasmid pPIC9K-*pvama-1* was linearized with SalI to transform the *P. pastoris* GS115 strain (*his4*$^-$) by electroporation. Approximately 350 His^+ clones transformed with the plasmid pPIC9K-*pvama-1* were screened for high-copy-number integration by G418 selection; of these clones, two were resistant to 2 mg/ml G418. Based on an immunoblotting analysis with mouse polyclonal anti-*Escherichia coli* PvAMA-1, a clone secreting high levels of PvAMA-1 and possessing a Mut^+ phenotype was selected.

The expression and purification of the recombinant protein PvAMA-1 were performed as previously described, with some modifications (28). A Mut^+ transformant was initially grown overnight in 200 ml BMGY medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 1.34% [wt/vol] yeast nitrogen base without amino acids, 4×10^{-5}% [wt/vol] biotin, 1% [wt/vol] glycerol, 0.1 M potassium phosphate [pH 6.0]) at 28 to 30°C with vigorous shaking. The cells were harvested, resuspended in 2 liters BMMY (BMGY with glycerol replaced by 0.5% [vol/vol] methanol), and incubated again for 72 h. Methanol was added at a final concentration of 1% (vol/vol) every 24 h. After induction for 72 h, the cells were removed by centrifugation, and the culture supernatant was concentrated by ultrafiltration with an Amicon Ultracel 30,000-molecular-weight-cutoff (MWCO) membrane (Millipore) and extensively dialyzed at 4°C against 20 mM sodium phosphate buffer (pH 8.0)–0.2 M NaCl. The supernatant was applied to a column with Ni^{2+}-nitrilotriacetic acid (NTA) agarose resin (Qiagen), which was previously equilibrated (20 mM sodium phosphate buffer [pH 8.0], 0.5 M NaCl). The bound proteins were eluted with a 15 to 400 mM imidazole (Sigma) gradient in wash buffer (20 mM sodium phosphate buffer [pH 8.0], 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10% glycerol). Fractions containing the protein were pooled and used in a second-step purification of anionic exchange chromatography using Q FF resin (GE Healthcare) coupled to ÄKTA Prime Plus (GE Healthcare). The protein was eluted using a 0 to 1 M NaCl linear gradient. The peak corresponding to PvAMA1 with a high degree of purity was collected and dialyzed against phosphate-buffered saline (PBS). The protein concentration was determined by the Bradford method (Bio-Rad) using bovine serum albumin (BSA) (Sigma) as the standard.

Purified PvAMA-1 was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Vydac C_4 column (4.6 mm by 250 mm; 300-μm particle size) and a Shimadzu LC solution HPLC system (Shimadzu Corp., Kyoto, Japan). The HPLC procedure was performed using an acetonitrile gradient from 0 to 100% in 0.1% trifluoroacetic acid (TFA)–90% acetonitrile at room temperature (≈24°C) at 1 ml/min for 40 min. The elution was monitored with a UV-visible absorbance detector (Shimadzu SPD M20A) at 214 nm.

The protocol for PvAMA-1 described above was validated in accordance with the requirements of "conditions of good laboratory practice" (cGLP) under contract with the Company Farmacore Biotecnologia, Ltd. (Ribeirão Preto, São Paulo, Brazil). In this system, the final product is subjected to analytical tests, SDS-PAGE and isoelectric focusing (IEF), Western blotting, bicinchoninic acid (BCA) quantification, endotoxin determination, and sterility. Vials containing the purified recombinant protein were stored at −80°C.

Expression and purification of the PvAMA-1 ectodomain in *E. coli* have been described elsewhere (23). The recombinant proteins were purified by Ni-affinity following published protocols.

CD spectroscopy. Circular dichroism (CD) spectroscopy was performed using a JASCO-J720 spectropolarimeter. Recombinant PvAMA-1 diluted to 9.96 μM in PBS was loaded into a 5-mm quartz cuvette. Far-UV measurements (8 scans) were performed over wavelengths of 260 to 200 nm with a 1-nm bandwidth, 1-s response time, and 20-nm/min scan speed. The spectra were corrected by subtraction of the buffer signal. The mean residue molar ellipticity, represented as $[\theta]_{MRW}$, was calculated (29), and the secondary structure was estimated by computer analysis using CDNN software (Applied Photophysics, Ltd.).

Recombinant plasmid used for immunizations. The gene encoding amino acids 43 to 487 of the PvAMA-1 ectodomain was obtained by PCR

using the plasmid pMOS-*ama-1* as the target DNA (23). The two synthetic oligonucleotide primers were 5′-GGAGGTACCCCTACCGTTGAGAGA AGC-3′ (forward) and 5′-AGTGGATCCTAGTAGCATCTGCTTGTTC GA-3′ (reverse) (Invitrogen). The oligonucleotide primers were synthesized with a KpnI (forward) or BamHI (reverse) restriction site (underlined). The resulting PCR amplification products were cloned into pGEM-T Easy (Promega), and positive clones were selected by DNA restriction endonuclease analysis and further confirmed by nucleotide sequence analysis. The *pvama-1* gene was removed from the pGEM-T Easy vector by digestion with KpnI and BamHI and cloned into the vector pIgSP (30) digested with the same enzymes. A colony was selected with a plasmid containing the insert in the correct orientation. This plasmid contains the sequence encoding the signal peptide of the mouse immunoglobulin kappa chain in the commercial vector pcDNA3 (Invitrogen). The plasmids were grown in *E. coli* DH5α and purified using the Qiagen Plasmid Giga kit. The DNA concentration was estimated at 260 nm.

Mice and immunization protocol. Female BALB/c (H-2^d) mice at 6 to 8 weeks old were used in all the experiments; the animals were purchased from the University of São Paulo, São Paulo, SP, Brazil. Study protocol no. 112 was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences of the University of São Paulo. The immunogenicity of the recombinant PvAMA-1 protein was evaluated in mice using homologous prime-boost protocols (DNA prime-DNA boost and protein prime-protein boost) and heterologous prime-boost strategies (DNA prime-protein boost or protein prime-DNA boost). For the DNA immunizations, the pIgSP empty (D^{ctrl}) or pIgSP-*ama-1* (D) plasmid was injected as previously described (30, 31). Briefly, both tibialis anterioris muscles were injected with 7 μg cardiotoxin (Sigma, St. Louis, MO); 5 days later, 100 μg plasmid DNA was injected intramuscularly at the same sites as the previous cardiotoxin injections (D*). The effect of DNA administration in the absence of cardiotoxin also was evaluated (D). Each mouse received four intramuscular doses of plasmid DNA injected at 0, 3, 6, and 9 weeks (D/D); in parallel, other groups of mice received the DNA prime-protein boost (D/P) or the protein prime-DNA boost (P/D). The immunizations with the recombinant protein (P) were performed by a subcutaneous (s.c.) route with 10 μg of the protein in the absence of any adjuvant or in the presence of incomplete Freund's adjuvant (IFA) (Sigma). A volume of 50 μl was injected into each footpad. For comparison, one group was immunized with the protein emulsified in complete Freund's adjuvant (CFA) (Sigma), and the animals received a booster injection of 10 μg of the same protein emulsified in IFA, injected s.c. at the base of the tail (P/P) after 3, 6, and 9 weeks. The controls received only PBS emulsified in adjuvant. Twenty days after each immunization, blood was collected from the tail, and the sera were analyzed for the presence of antibodies against PvAMA-1 recombinant protein.

Subsequently, the immunizations were performed with the recombinant PvAMA-1 protein formulated in different adjuvants, including 100 μg Alhydrogel (Superfos Biosector, Denmark), 10 μg *Bordetella pertussis* monophosphoryl lipid A (MPLA), 5 μg flagellin FliC from *Salmonella* enterica serovar Typhimurium, 25 μg saponin Quil A (Superfos Biosector, Denmark), and incomplete Freund's adjuvant (IFA). MPLA was produced using lipopolysaccharide (LPS) from previously detoxified whole-cell pertussis vaccine, as previously described (32). The PvAMA-1 protein was also coadministered in aluminum salts (Alum) plus FliC or Alum plus MPLA. These adjuvants were administered at the same doses used for the immunizations with single adjuvants. The controls received only PBS emulsified in adjuvant. The immunization schedule was the same as that used in the heterologous prime-boost protocol, except that the interval between the doses was 2 weeks for all of the different adjuvant formulations. In summary, groups of BALB/c mice were immunized with one of the following regimens: (i) 4 doses of plasmid DNA (pIgSP-*pvama-1*) without (D/D) and with cardiotoxin (D*/D); (ii) 4 doses of PvAMA-1 protein in the absence or presence of CFA/IFA (P/P); (iii) one dose of PvAMA-1 protein in the absence or presence of IFA, followed by 3 doses of pIgSP-*pvama-1* (P/D); (iv) one dose of pIgSP-*pvama-1* immunization, followed by 3 doses of PvAMA-1 protein in the presence of IFA (D*/P); or (v) one dose of plasmid pIgSP followed by 3 doses of PvAMA-1 protein emulsified in IFA (D^{ctrl}*/P).

Immunological assays. (i) Immunoblotting analysis. Protein fractions were fractionated by 12% SDS-PAGE under reducing or nonreducing conditions and transferred from the gel to nitrocellulose membranes (Hybond N; GE Healthcare) with the aid of a Mini Trans-Blot apparatus (Bio-Rad). The membranes were saturated for 2 h at room temperature in PBS-milk-BSA (5% [wt/vol] nonfat milk and 2.5% [wt/vol] bovine serum albumin). The membranes were then incubated with a mouse monoclonal anti-histidine (anti-His) tag (GE Healthcare) at a final dilution of 1:1,000 or with monoclonal antibodies (MAbs) against PvAMA-1 domain II (33). After 1 h at room temperature, the membranes were washed three times with PBS–0.05% (vol/vol) Tween 20 (PBS-Tween), and goat anti-mouse IgG coupled to peroxidase was added to the membranes at a final dilution of 1:2,000 (KPL, Gaithersburg, MD). After 1 h of incubation at room temperature, the reaction was developed using the ECL enhanced chemiluminescence detection assay (GE Healthcare).

(ii) Detection of human anti-PvAMA-1 antibodies by ELISA. Serum samples were previously collected from 208 individuals with patent *P. vivax* malaria living in regions of endemicity in the state of Pará (northern Brazil). Clinical and laboratory data have been reported elsewhere for all individuals (34). The study protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences of the University of São Paulo (CEP no. 354/2006). The detection of human IgG antibodies specific for the recombinant proteins was performed by enzyme-linked immunosorbent assay (ELISA), as described previously (26). The ELISA plates were coated with 100 ng/well of PvAMA-1; for comparison, a bacterial recombinant protein previously produced (23, 26) was also tested against the same sera. A 50-μl aliquot of each solution was added to each well of Costar high-binding 96-well plates. After an overnight incubation at room temperature, the plates were washed with PBS-Tween and blocked with 5% nonfat milk in PBS (PBS-milk) for 2 h at 37°C. Serum samples were added to each well in duplicate at a final dilution of 1:100. After incubation for 2 h at room temperature and washes with PBS–0.05% Tween 20, we added 50 μl of a solution containing peroxidase-conjugated goat anti-human IgG (Fc specific; Sigma) at a final dilution of 1:5,000 to each well. The enzymatic reaction was developed with *o*-phenylenediamine (1 mg/ml) (Sigma) diluted in phosphate-citrate buffer (pH 5.0) containing hydrogen peroxide (0.03% [vol/vol]). The enzymatic reaction was stopped by the addition of 50 μl of a solution containing 4 N H_2SO_4. The optical density at 492 nm (OD_{492}) was measured using an ELISA reader (Awareness Technology, model Stat Fax 3200, EUA). The cutoff points were set at 3 standard deviations (SD) above the mean OD_{492} value of the sera from 25 healthy individuals from the city of São Paulo who had never been exposed to malaria (34).

(iii) Determination of mouse antibody titers against PvAMA-1. Antibodies against PvAMA-1 in mouse sera were detected by ELISA, essentially as described previously (26, 33). The recombinant PvAMA-1 proteins were employed as solid-phase-bound antigen (100 ng/well), and a volume of 50 μl of each solution was added to each well of 96-well plates. After overnight incubation at room temperature, the plates were washed with PBS-Tween and blocked with PBS-milk–2.5% BSA for 2 h at 37°C. The polyclonal sera of the mice were tested at serial dilutions in a final volume of 50 μl of sample added to each well in duplicate, followed by incubation for 1 h at room temperature. After washes with PBS-Tween, 50 μl of a solution containing the secondary antibody conjugated to peroxidase (goat anti-mouse IgG; KPL) diluted 1:3,000 was added to each well. The enzymatic reaction was developed as described above. The anti-PvAMA-1 titers were determined as the highest dilution yielding an OD_{492} higher than 0.1. The results are expressed as means of IgG titers

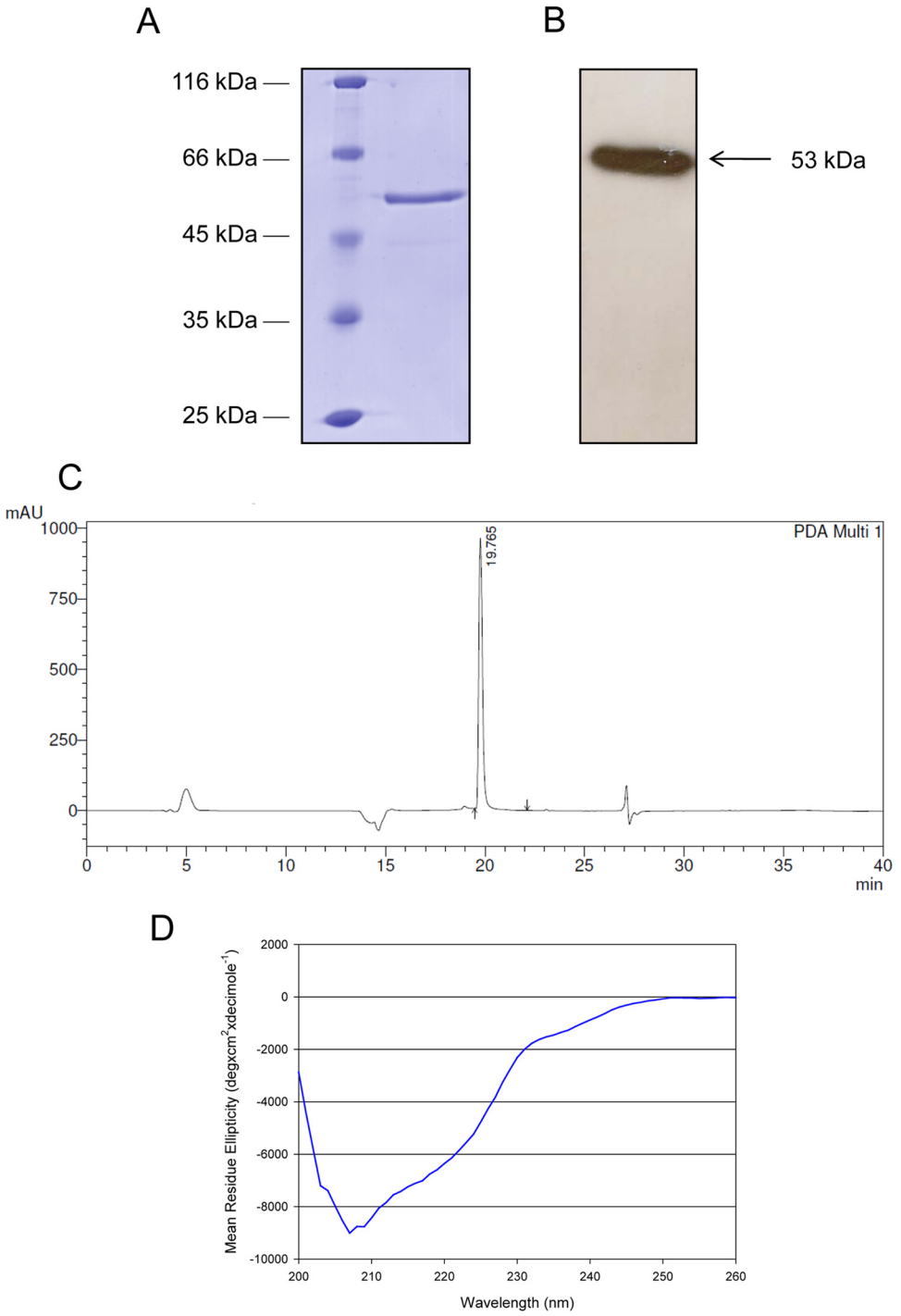


FIG 1 The recombinant protein was expressed in *P. pastoris* with a hexahistidine tag and purified from the supernatant by affinity chromatography, followed by anion-exchange chromatography, as described in Materials and Methods. (A) SDS-PAGE analysis of purified recombinant PvAMA-1 stained with Coomassie blue (1 μg of protein per lane). (B) Immunoblotting analysis of purified recombinant PvAMA-1 using an anti-histidine tag antibody. (C) RP-HPLC profile of purified recombinant PvAMA-1. Purified PvAMA-1 produced under cGLP was analyzed by RP-HPLC using a C_4 column, as described in Materials and Methods. (D) Circular dichroism spectrum of recombinant PvAMA-1. The spectrum was recorded from 200 to 260 nm using a JASCO-J720 spectropolarimeter. The plot represents the mean residue ellipticity of the recombinant protein.

($\log_{10}$) ± standard errors of the mean (SEM) detected at 2 weeks after each immunizing dose.

(iv) Indirect immunofluorescence assay. Immunofluorescence assays were performed as described previously (27). Merozoite preparations were made from *ex vivo* matured and concentrated schizonts as described in the section v below. The thin-smear preparations of free merozoites and mature schizont-infected erythrocytes were fixed with cold acetone for 15 min and blocked with 3% BSA in PBS for 30 min at 37.5°C in a humidified

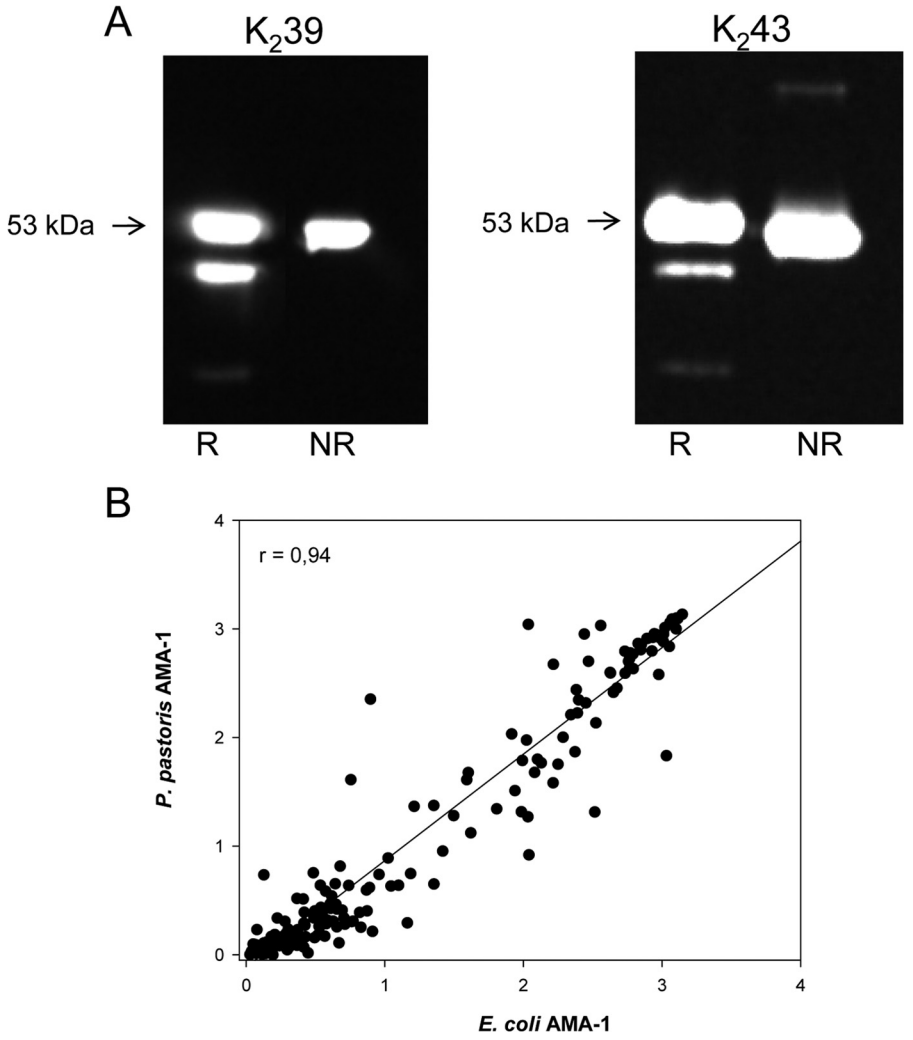


FIG 2 Immunoblotting analysis of recombinant PvAMA-1 using monoclonal antibodies and a comparative evaluation of PvAMA-1 expressed in *E. coli* or *P. pastoris* for recognition by human sera. (A) Recombinant PvAMA-1 was subjected to 12% SDS-PAGE performed under reducing (R) and nonreducing (NR) conditions. Immunoblotting was performed using the indicated monoclonal antibodies. (B) Sera from 208 individuals with patent *P. vivax* malaria from areas of endemicity in Brazil were tested against recombinant PvAMA-1 produced in *E. coli* or *P. pastoris*. The values are the OD_{492} measurements of each protein. The tendency line and the value of the Spearman correlation coefficient (*r*) are represented.

incubator. Sera from animals immunized with AMA-1 plus Quil A or AMA-1 plus MPLA (dilution, 1:100) were applied to the slides and incubated for 1 h. The slides were then washed 3 times with PBS before the addition of anti-mouse IgG conjugated to Alexa Fluor 568 (Molecular Probes) diluted 1:500 with 3% BSA in PBS or DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen). Binding was visualized using a Nikon TS 100 epifluorescence microscope.

(v) Inhibition invasion assay. Four clinical isolates from *P. vivax*-infected blood from malaria patients attending the clinics of the Shoklo Malaria Research Unit (SMRU), Mae Sot region northwest of Thailand, were collected after written informed consent (OXTREC 027-025; University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, Oxford, United Kingdom). These blood samples were collected by venipuncture in 5-ml-volume lithium heparinized tubes, which were transported to the laboratory at SMRU within 5 h of collection. White blood cells and platelets were removed using a CF11 column (35). The *P. vivax*-infected erythrocytes were cultured to the late schizont stage in 2% hematocrit (Hct) McCoy's 5A medium supplemented with 2.4 g/liter D-glucose, 40 mg/ml gentamicin sulfate, and 20% heat-inactivated human AB serum, in an atmosphere of 5% O_2 at 37.5°C for ~44 h. The mature schizonts were concentrated on a cushion of 45% Percoll (isotonic) centrifuged for 15 min at 1,600 × *g* (36). After being washed twice in McCoy's 5A medium, thin-smear preparations of the schizont concentrate were split into two portions (~5 μl each). The first portion was smeared onto glass slides, air dried, and fixed with cold acetone for 15 min and stored at −20°C until needed (These were used in the "Indirect fluorescence assay" section above). The remainder of the schizont concentrate was then utilized in a *P. vivax* invasion assay that utilized reticulocytes enriched from one isolate of human cord blood (37). In addition to the treatment (AMA-1 plus Quil A [1:100]) and untreated control, it is vital to use the positive control (25 μg/ml of antibody 2C3, a monoclonal antibody against the Duffy antigen receptor [DARC]), which almost always blocks *P. vivax* invasion in this isolate (a kind gift from Yves Colin and Olivier Bertrand, INSERM UMR-S665 and Institut National de la Transfusion Sanguine, Paris, France). To provide for an objective and quantitative measure of *P. vivax* invasion in the treatments and controls after 24 h of incubation, we used the tricolor flow cytometry method using a field-deployable flow cytometer (BD Accuri C6 flow cytometer) (38). In addition, the flow cytometry data were cross-checked with Giemsa-stained smears using microscopy.

Statistical analysis. Correlations were determined by the nonparametric Spearman correlation coefficient. A one-way analysis of variance (ANOVA) was used to compare possible differences in the mean values, with the level of significance set at $P < 0.05$.

Nucleotide sequence accession number. The sequence for yPvAMA-1 was deposited into GenBank under accession number KJ010958.

RESULTS

Expression, purification, and biochemical characterization of recombinant PvAMA-1. To improve protein expression, we used a codon-optimized gene for secreted expression in the methylotrophic yeast *P. pastoris* based on the previously described *pvama-1* sequence (23). Only the ectodomain of PvAMA-1 was synthesized, representing amino acids 43 to 487. This amino acid sequence has three putative N-linked glycosylation sites, and we performed conservative mutations to remove these sites (178N→S, 226N→D, and 441N→Q) using substituent amino acids from available AMA-1 sequences of other malaria parasites (10). The final construct also encodes six amino acids that include the N-terminal hexahistidine tag for Ni^{2+}-chelating chromatography. The synthetic *pvama-1* gene was subcloned into the commercial expression vector pPIC9K in frame with the nucleotides encoding the yeast α-factor secretion signal peptide.

The protein was expressed as a secreted, soluble protein, and the yield (7 mg/liter) was superior to that obtained previously in *E. coli* (23). The protein was purified as described in Materials and Methods, and the final protein purity was >90% according to SDS-PAGE and Coomassie blue staining, which revealed a predominant band that migrated at approximately 53 kDa under reducing conditions (Fig. 1A). Using an immunoblotting analysis, the protein was detected by an anti-His tag MAb, indicating that the His_6 tag had been preserved (Fig. 1B).

The homogeneity of the recombinant protein was confirmed by reverse-phase chromatography on a C_4 column, and a single peak was observed, as shown in Fig. 1C. To investigate the folding of recombinant PvAMA-1, we examined the protein by far-UV CD spectroscopy (Fig. 1D). The CD data were deconvoluted using the CDNN program (Applied Photophysics, Ltd.), and the percentages of α-helix, anti-parallel β-sheet, parallel β-sheet, β-turn, and random coil were estimated. The PvAMA-1 CD spectrum is consistent with a folded protein and predicts approximately 12.4% α-helix, 20.7% anti-parallel β-sheet, 19.6% parallel β-sheet, 22.5% β-turn, and 54.2% random coil structures. These data are consistent with the secondary structure of PvAMA-1 (strain SalI) crystallized by Pizarro et al. in 2005 (39).

Antigenic characterization. Immunoblotting probed with two anti-PvAMA-1 domain II MAbs which recognize fixed parasites by IFA (33) showed that they reacted strongly with PvAMA-1 under reducing or nonreducing conditions (MAbs $K_2$39 and $K_2$43), suggesting that these MAbs recognize nonconformational epitopes associated with the entire PvAMA-1 antigen (Fig. 2A). The same results were obtained with MAbs $K_2$14, $K_2$68, and $K_2$78 (data not shown).

Purified PvAMA-1 produced in *E. coli* and *P. pastoris* were examined by ELISA with sera collected from 208 individuals during patent *P. vivax* infection (25). The results showed that 72.7% and 62.5% of the sera recognized *E. coli* PvAMA-1 and *P. pastoris* PvAMA-1, respectively. A high Spearman correlation coefficient (r) was obtained when we compared the reactivities of human antibodies to both recombinant proteins ($r = 0.94$), indicating that the recombinant protein produced in yeast maintains its antigenic properties (Fig. 2B).

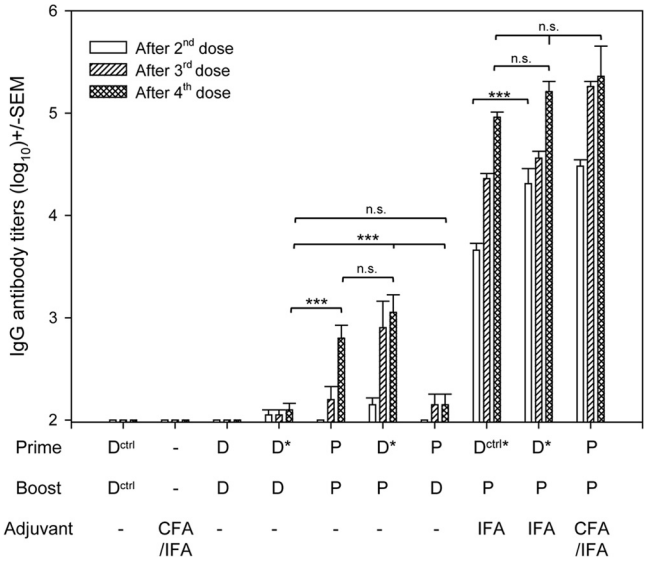
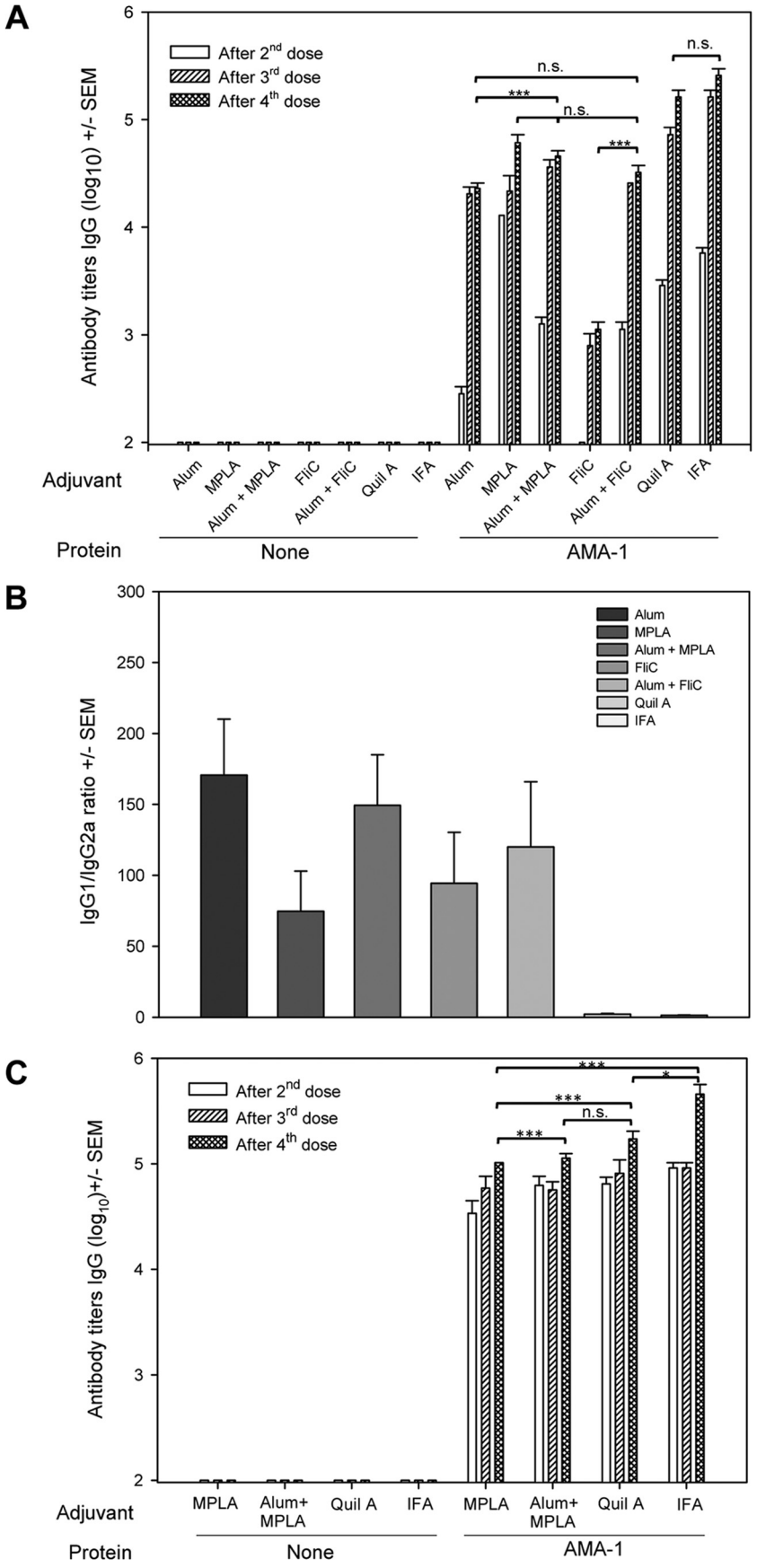


FIG 3 Serum IgG titers of mice immunized with recombinant PvAMA-1 and/or plasmid DNA containing the *pvama-1* gene. Mice were immunized 4 times with 100 μg of DNA (D) i.m. or 10 μg of recombinant protein (P) s.c. with a 20-day interval. The protein was administered without adjuvant or emulsified in CFA/IFA. Five days prior to immunization with DNA, some groups received cardiotoxin (D*). The animals from control groups were immunized with empty DNA vector (D^{ctrl}) or CFA/IFA diluted in PBS. The results are expressed as the mean $\log_{10}$ titers ± SEM. n.s., nonsignificant ($P > 0.05$). ***, $P < 0.001$.

Immunogenic properties. Mice were immunized using different schedules to provide a direct comparison of the relative immunogenicities of homologous (D/D or P/P) versus heterologous (D/P or P/D) prime-boost vaccinations. Groups of BALB/c mice were immunized with the following: (i) plasmid DNA (pIgSP-*pvama-1*) without (D/D) and with cardiotoxin (D*/D), (ii) PvAMA-1 protein in the absence or presence of CFA/IFA (P/P); (iii) PvAMA-1 protein in the absence or presence of IFA followed by pIgSP-*pvama-1* (P/D), (iv) pIgSP-*pvama-1* immunization, followed by PvAMA-1 protein in the presence of IFA (D*/P), and (v) plasmid pIgSP followed by PvAMA-1 protein emulsified in IFA (D^{ctrl}*/P).

The first two schedules are homologous prime boosting using the same type of vaccine. The third and fourth schedules are heterologous prime boosting with DNA or recombinant protein administered for priming, followed by three heterologous booster immunizations. Two additional immunization schedules were included as controls. The mice received only plasmid DNA vector (pIgSP) immunizations (D^{ctrl}*/D^{ctrl}), and another control group received only PBS in CFA/IFA.

The comparative immunogenicity in each animal group was determined by the mean of the serum IgG antibody titers of each individual mouse against the PvAMA-1 antigen estimated by ELISA. After 4 immunizing doses with the different immunization protocols, we observed that the D*/P (IFA) and P/P (CFA/IFA) protocols induced significantly higher serum IgG antibody titers than the D*/D, P/D, and P/P (without adjuvant) regimens (Fig. 3). These results showed that the efficiency of the PvAMA-1 immunization is highly dependent on a formulation containing the re-


A
Antibody titers IgG (log10) +/- SEM
After 2nd dose
After 3rd dose
After 4th dose
n.s.
***
n.s.
***
n.s.
Adjuvant
Alum
MPLA
Alum + MPLA
FliC
Alum + FliC
Quil A
IFA
Protein
None
AMA-1
B
IgG1/IgG2a ratio +/- SEM
Alum
MPLA
Alum + MPLA
FliC
Alum + FliC
Quil A
IFA
C
Antibody titers IgG (log10)+/- SEM
After 2nd dose
After 3rd dose
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*
Adjuvant
MPLA
Alum+ MPLA
Quil A
IFA
Protein
None
AMA-1

combinant protein in adjuvant. In spite of the observation that the mice receiving the DNA prime immunization followed by booster immunization with PvAMA-1 protein in IFA (D*/P) presented slightly higher antibody responses than those of the control group (D^{ctrl}*/P), the endpoint serum dilution titers among these groups were not statistically different after the second dose. Thus, we concluded that priming with plasmid DNA did not improve vaccination (Fig. 3). The animals that received immunizations with only the empty DNA vaccine vector (D^{ctrl}*/D^{ctrl}) or pIgSP-*pvama-1* without cardiotoxin (D/D) did not present specific antibodies against PvAMA-1. Additionally, the recombinant protein alone showed negligible immunogenicity in the absence of adjuvant, highlighting the extreme importance of the choice of adjuvant in the formulation of malaria vaccines used in preclinical immunization against *P. vivax*.

The identification of potent adjuvants capable of inducing antibody responses as high as Freund's adjuvant is considered a great challenge in the development of subunit vaccines for malaria. Thus, we evaluated the immunogenicity of PvAMA-1 in mice in the presence of other adjuvants, including aluminum salts (Alum), *Bordetella pertussis* MPLA, flagellin FliC from *Salmonella* Typhimurium, saponin Quil A, and IFA, for comparison. BALB/c mice were immunized subcutaneously with each formulation, and the IgG antibodies titers against PvAMA-1 were detected by ELISA 15 days after each dose. At the end of the immunization schedule, we observed that the formulation containing Quil A or IFA displayed higher serum IgG antibody titers (5.21 ± 0.06 and 5.41 ± 0.06, respectively) (Fig. 4A). No statistically significant differences in the mean antibody titers were detected among the mice immunized with formulations containing PvAMA-1 and Quil A or IFA ($P > 0.05$, one-way ANOVA). Lower but still significant antibody titers were obtained using Alum, MPLA, Alum plus MPLA, or Alum plus FliC (4.36 ± 0.05, 4.78 ± 0.07, 4.66 ± 0.05, or 4.51 ± 0.06, respectively). The lowest antibody titers were obtained using the protein coadministered in FliC (3.05 ± 0.07). The comparison of the mean values of the antibody titers of the mice immunized with PvAMA-1 in the presence of MPLA or MPLA plus Alum did not reveal a statistically significant difference among them ($P > 0.05$, one-way ANOVA). Moreover, the use of MPLA alone, instead of in combination with Alum, reduced the IgG1/IgG2a ratios from 794 to 125 (Fig. 4B). As expected, IgG1 isotypes predominated in the sera of the animals immunized with the formulation containing PvAMA-1 and Alum (Alum alone, Alum plus MPLA, or Alum plus FliC). In contrast, more balanced IgG isotype responses were induced with the formulations containing Quil A or IFA, characterizing a mixed Th1-Th2 response (Fig. 4B).

To confirm that our recombinant protein could be further used for vaccine development, PvAMA-1 was produced under "conditions of good laboratory practice" (cGLP) by the company Farmacore Biotecnologia, Ltd. Following our protocol, a batch of protein with a good yield, low endotoxin levels, and high purity was obtained (Table 1). The results of experimental immunizations using this antigen in the presence of selected adjuvants (MPLA, Quil A, and IFA) confirmed the high immunogenicity of PvAMA-1 (Fig. 4C).

TABLE 1 Characteristics of the final product of the PvAMA-1 vaccine obtained under cGLP

Characteristic	Result	Method
Concn	1.69 mg/ml	Micro-BCA protein assay kit
Purity by SDS-PAGE	>85%	Phast System (GE Healthcare)
Molecular size	Single 52-kDa band	SDS-PAGE
Binding to monoclonal anti-His	Positive	Western blot
Endotoxin content	7.21 EU[a]/mg	Kinetic-QCL kit
Sterility	Sterile	Bioburden test
Product potency	Induction of high IgG antibody titers ($\log_{10}$, >5) in mice following s.c. immunization	ELISA

[a] EU, endotoxin units.

In addition, the sera from mice immunized with PvAMA-1 in formulations containing MPLA or Quil A (Fig. 5) and Alum or Alum plus MPLA (see Fig. S1 in the supplemental material) were able to recognize the native protein exposed on the surface of *P. vivax* merozoites isolated from infected donors from Thailand. To evaluate the ability of the antibodies raised against PvAMA1 to inhibit the reinvasion of new erythrocytes, we used four different isolates from infected donors from Thailand and tested a pool of sera from mice immunized with PvAMA-1 (cGLP) in a formulation containing Quil A. The results showed successful inhibition, ranging from 10.79% to 43.38% compared to the prebleed serum negative control (Fig. 6).

DISCUSSION

In previous studies, we showed that recombinant proteins expressed in *E. coli* based on the *P. vivax* AMA-1 sequence were recognized by IgG antibodies of a large fraction of malaria-infected individuals (23–26). We also described that these recombinant proteins can induce high antibody titers in mice following a homologous (protein-protein) or heterologous (protein-adenovirus) regimen of vaccination (27). Unfortunately, when produced in *E. coli*, the ectodomain of PvAMA-1 is insoluble and requires denaturation and refolding for purification (23, 26). Thus, to attempt to solve this problem, the present study was designed to express a soluble form of the ectodomain of PvAMA-1 as a secreted polypeptide in the yeast *P. pastoris*.

Using this system, we successfully generated a soluble antigenic protein under cGLP that exhibited a high degree of purity and low endotoxin and microbial contents. The yeast-derived PvAMA-1 protein retained its antigenicity, as it was recognized by the IgG

FIG 4 Serum IgG titers of mice immunized with recombinant PvAMA-1 using formulations containing different adjuvants. (A) Groups of BALB/c mice were immunized s.c. with 10 μg of the PvAMA-1 protein in the presence of different adjuvants, as described in Materials and Methods. These adjuvants were tested alone or in combination (Alum + MPLA and Alum + FliC). The results are expressed as the mean $\log_{10}$ titers ± SEM. n.s., nonsignificant ($P > 0.05$). ***, $P < 0.001$. (B) Comparative serum IgG subclass profile after mouse immunization with PvAMA-1 using different adjuvants. The results are expressed as the IgG1/IgG2a ratio of titer ± SEM. (C) Immunogenicity of the PvAMA-1 protein produced under "conditions of good laboratory practice" (cGLP). The results are expressed as mean $\log_{10}$ antibody titers ± SEM. n.s., nonsignificant ($P > 0.05$); *, $P < 0.05$; ***, $P < 0.001$.

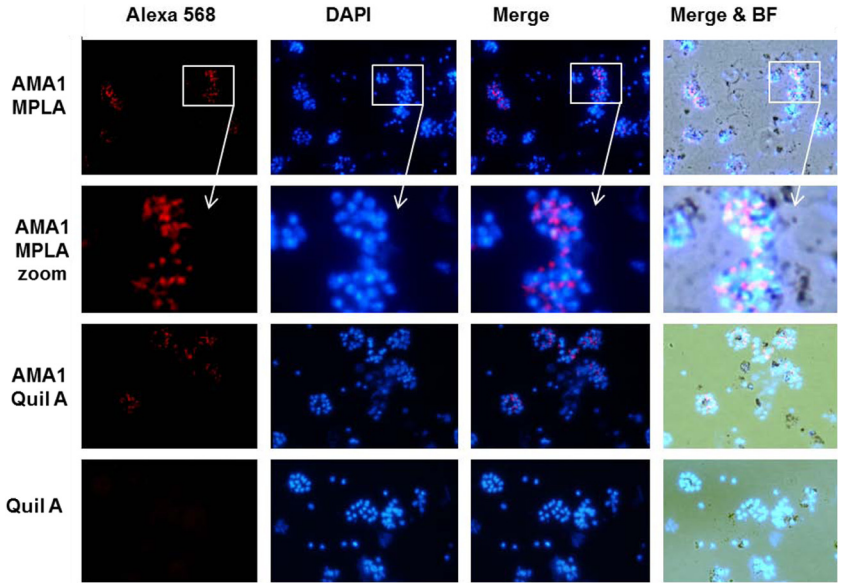

FIG 5 Indirect immunofluorescence analysis using sera from BALB/c mice immunized with the cGLP PvAMA-1 protein in formulations containing different adjuvants. The second row of panels shows a magnified region of the corresponding panels above. These magnified images highlight the apical staining pattern of anti-PvAMA-1 plus MPLA. Microscope slides containing fixed *P. vivax* cells obtained from patients from Thailand were incubated with sera from mice immunized with PvAMA-1 in the presence of MPLA or Quil A (diluted 1:100). BF, bright field.

antibodies from 62.5% of individuals infected with *P. vivax*. The recognition was comparable to the *E. coli*-derived PvAMA-1 recombinant protein ($r = 0.94$), indicating the presence of epitopes in both proteins shared with native PvAMA-1.

The evaluation of immunogenicity in mice showed that IFA and Quil A induced higher IgG titers and a more balanced Th1/Th2 response. Relevant for vaccine development was the observation that lower but also significant titers were obtained in the presence of the adjuvants licensed for human use: Alum, MPLA, or the combination of Alum plus MPLA. Formulations with the adjuvants Alum or Alum plus MPLA predominantly induced a Th2 response, and the use of MPLA alone balanced this response. Formulations containing MPLA can be used for human vaccination trials, and the MPLA we used was recently developed and investigated in human trials as an adjuvant for an influenza vaccine (40). Formulations containing either MPLA, Alum, and squalene or MPLA plus Alum have been proven to be safe and immunogenic for humans (40).

Although high antibody titers are important, protective immunity against infection will only be achieved if these antibodies recognize the native protein and inhibit parasite invasion of reticulocytes. The immunofluorescence results confirmed the recognition of native protein from *P. vivax* isolates from Thailand by the sera of immunized mice. Because our protein was based on an Amazonian isolate of *P. vivax*, these results reflect the presence of cross-reactive epitopes.

It has long been known that antibodies against *Plasmodium* AMA-1 have a strong invasion-inhibitory activity (3). However, one of the greatest limitations for *P. vivax* vaccine development has been the lack of a functional *in vitro* assay to routinely assess the invasion-inhibitory activity of the antibodies. To overcome this limitation, we took advantage of a recently described *ex vivo* reinvasion assay (37) to test whether the sera of mice immunized with PvAMA-1 presented invasion-inhibitory activity. We found that the antibodies obtained from mice immunized with PvAMA-1 in the presence of Quil A inhibited the reticulocyte invasion of four different isolates from Thailand, results that were for the first time obtained with *P. vivax*. Our results of parasite inhibition are compatible with our own previous studies using immune IgG against region II of the Duffy binding protein (37). Essentially, these findings confirm and extend the previous studies of other species of *Plasmodium*, providing further support for the implementation of PvAMA-1 as a vaccine candidate against *P. vivax* malaria.

However, the main problem with using AMA-1 as a malaria vaccine component is the known allelic polymorphism, which may generate allele-specific invasion-inhibitory antibodies. In fact, as mentioned in the introduction, the results obtained in a phase II trial using the vaccine FMP2.1/AS02A, a recombinant protein of PfAMA-1 based on the 3D7 allele, strongly argue in favor of the interpretation of strain-specific resistance against malaria infection (20).

Obviously, because *P. vivax* research has been highly neglected, to our knowledge, nothing is known regarding the immunological impact of the allelic differences of PvAMA-1. Indeed, it is unknown whether a similar strain-specific immunity will be induced by vaccination with the recombinant protein we described herein. Therefore, it would not be proper to speculate at this time.

In spite of the problems faced by allelic polymorphisms, the potential of AMA-1 as a vaccine component against *P. falciparum* malaria continues to attract a number of important research groups in this field. These laboratories are investing different approaches to overcome the problem imposed by the allelic polymorphism of PfAMA-1 (41–43). In addition, recent studies using parasites isolated from individuals vaccinated with FMP2.1/AS02A mapped few amino acid variations as the cause of strain-specific resistance (44). Such limited polymorphism raises the

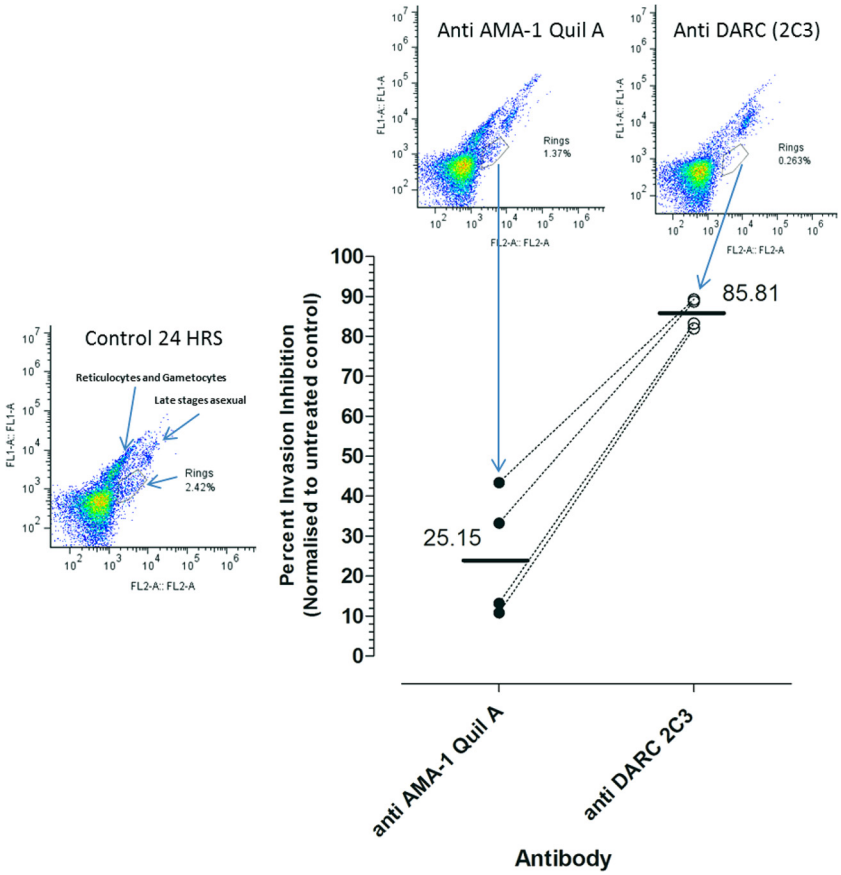

FIG 6 Invasion-inhibitory activity of sera from mice immunized with PvAMA-1 (cGLP) in the presence of Quil A (1:100) against merozoites from four Thai isolates of *P. vivax* (mean ± SD inhibition, 25.15% ± 15.77%). A flow cytometry method using a field-deployable flow cytometer (BD Accuri C_6 flow cytometer) was used to quantify the amount of invasion inhibition relative to the treatment free control (1:100 prebleed mouse sera) (FL1-A with SYBR green and FL2-A with dihydroethidium). Each assay was compared to a positive control (anti-DARC [2C3 region]) (mean ± SD inhibition, 85.81% ± 3.72%). Surrounding the central graph, we provide the fluorescence-activated cell sorter (FACS) scatter plots of the inhibition assays. The FACS plot on the left side of the *y* axis shows a gate containing 2.42% rings (postinvasion) in the untreated control (1:100 prebleed mouse sera) compared to 1.37% and 0.26% in the AMA-1 treatment and DARC positive control, respectively.

possibility that a few recombinant proteins representing key alleles would cover the entire population of *P. falciparum*. In the worst-case scenario, if similar problems of strain-specific immunity arise due to allelic polymorphism of the PvAMA-1 protein, perhaps solutions similar to those developed for *P. falciparum* AMA-1 can be adopted for *P. vivax*.

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